host plant at 24 h before inoculation. Inoculation was carried out by spraying the conidia of *Pyricularia oryzae* Cavara [rice blast, anamorph of *Magnaporthe grisea* (Hebert) Barr], *Sphaerotheca fuliginea* (Schlecht) Poll (cucumber powdery mildew) or *Erysiphe graminis* DC f sp *tritici* Marchal (wheat powdery mildew) onto the host plants, or by putting the mycelial mats of *Botrytis cinerea* Pers (cucumber gray mold), or dropping a zoosporangial suspension of *Pseudoperonospora cubensis* (Berk & MA Curtis) Rost (cucumber downy mildew) onto the leaf surface of cucumber seedlings. The results in Table 1 suggested that the substituents on the benzene ring play an important role in fungicidal activity. Mono-, di- or tri-substitutions at the 2-, 3-, 4- and/or 5-positions of the benzene ring by halogen or lower alkyl also led to an increase in the activity. However, 2,6-di- or 2,3,6-tri-substituted derivatives were less active against all diseases tested.

Furthermore, the phenoxymethyl group could be replaced with a 2-pyridyloxymethyl group (**11**, Fig 1). Mono- or di-substitutions at the 3-, 5- and/or 6-position of the pyridine ring by Cl or CF_3 resulted in excellent control against a wide range of diseases of upland crops by foliar application.

Strobilurin analogues are a new class of fungicide with broad-spectrum activity and a new mode of action.[3–5] They inhibit the bc_1 segment of the respiratory chain of mitochondria.[6] SSF-126 also inhibits the same target site of mitochondria.[7] During the optimization process with the methoxyiminophenylacetamide derivatives, we noted the similarity of the isoxazole ring-cleaved compounds to the strobilurin analogues, derived from strobilurin A which is produced by a species of Basidiomycotina. Consequently, the methoxyiminophenylacetamide derivatives obtained by the approach of the ring-cleavage design of isoxazole are strobilurin analogues from the viewpoints of chemical structure and biological performance.

REFERENCES

1 Hayase Y, Ozaki M, Takeuchi Y and Kobayashi S, Reductive cleavage of the isoxazole ring under soil percolation *Nihon Noyaku Gakkashi* (*J Pestic Sci*) **7**:401–402 (1982).

2 Hayase Y, Kataoka T, Masuko M, Niikawa M, Ichinari M, Takenaka H, Takahashi T, Hayashi Y and Takeda R, Phenoxyphenyl alkoxyiminoacetamides: new broad spectrum fungicides, in *ACS Symposium Series 584. Synthesis and Chemistry of Agrochemicals IV*, ed by Baker DR, Fenyes JG and Basarab GS, American Chemical Society, Washington DC. pp 343–353 (1995).

3 Beautement K, Clough JM, de Fraine PJ and Godfrey CRA, Fungicidal β-methoxyacrylates: from natural products to novel synthetic agricultral fungicides. *Pestic Sci* **31**:499–519 (1991).

4 Ammermann E, Lorenz G, Schelberger K, Wenderoth B, Sauter H and Retzea C, BAS-490F: A broad-spectrum fungicide with a new mode of action. *Proc Brighton Crop Prot Conf Pests and Diseases*. pp 403–410 (1992).

5 Beautement K, Clough JM, de Fraine PJ and Godfrey CRA, Fungicidal β-methoxyacrylates, *N*-linked pyrroles, in *ACS Symposium Series 584. Synthesis and Chemistry of Agrochemicals IV*, ed by Baker DR, Fenyes JG and Basarab GS, American Chemical Society, Washington DC. pp 326–342 (1995).

6 Becker WF, von Jagow G, Anke T and Steglich W, Oudemansin, strobilurin A, strobilurin B and myxothiazole: new inhibitors of the bc_1 segment of the respiratory chain with an *E*-β-methoxyacrylate system as common structural element. *FEBS Lett* **132**:329–333 (1981).

7 Mizutani A, Yukioka H, Tamura H, Miki N, Masuko M and Takeda R, Respiratory characteristics in *Pyricularia oryzae* exposed to a novel alkoxyiminoacetamide fungicide. *Phytopathology* **85**:306–311 (1995).

Glutathione *S*-transferases in black-grass (*Alopecurus myosuroides* Huds.): Properties and involvement in herbicide resistance

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Abstract: Black-grass (*Alopecurus myosuroides* Huds) is a major problem weed in winter cereal crops in the UK. In recent years herbicide resistance has been detected in this species. Research suggests that this is due, at least in part, to increased herbicide metabolism. One group of enzymes implicated in herbicide metabolism are glutathione *S*-transferases (GSTs). In this study, GSTs have been purified from black-grass in order to investigate further their role in resistance. In addition, the effects of herbicides on GST activity *in vitro* have been studied.

Keywords: herbicide resistance; black-grass; *Alopecurus myosuroides*; glutathione *S*-transferase; protein purification

Glutathione *S*-transferases (GSTs. E.C.2.5.1.18) are a group of enzymes which catalyse the conjugation of a wide range of substrates with the tripeptide glutathione (γ-Glu-Cys-Gly; GSH). GSTs occur throughout the animal and plant kingdom. In animals they have been extensively studied and have been implicated in many roles including toxin and drug metabolism. In plants, GSTs have been less well studied, but have been implicated in herbicide resistance, crop herbicide tolerance, various stress responses and in secondary metabolism, as recently reviewed by Marrs.[1] GSTs have been linked to the metabolism of a variety of herbicides and, in the case of atrazine, glutathione conjugates have been isolated.[2] With this in mind, we have studied the possible roles played by GSTs in herbicide resistance in

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the arable grass weed *Alopecurus myosuroides* Huds (black-grass). This is a problem weed in winter cereal crops in the UK, causing substantial reductions in crop yield. Historically, it has been controlled by the use of both cultural and chemical means. However, due to over-reliance on monoculture and extensive use of herbicides with a single mode of action, resistance to herbicides has developed. This was first reported in the early 1980s in a black-grass population from Peldon, Essex, UK[3] which was demonstrated to be resistant to chlorotoluron (CTU). This biotype has subsequently been shown to have both multiple resistance and cross-resistance to a variety of herbicides.[4] Since the discovery of this biotype, many populations of black-grass exhibiting resistance to herbicides have been characterised in the UK. Research suggests that increased herbicide metabolism may be implicated in many cases. Indeed, study of the Peldon biotype has revealed approximately double GST activities compared to those in susceptible biotypes.[5] We have further investigated the roles that GSTs play in herbicide resistance in black-grass.

A purification procedure for GSTs from the susceptible black-grass biotype Herbiseed has been established in this laboratory.[6] GST activities were assayed at each stage using the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB). Tissue was ground to a powder in liquid nitrogen, homogenised in buffer and clarified by centrifugration. After protein harvesting by ammonium sulfate precipitation and subsequent dialysis, proteins were separated by ion-exchange chromatography using a 2-ml anion exchange column (2 × 1 ml Econo-Pac High Q. Bio-Rad Laboratories Ltd.). One peak of protein showing GST activity was eluted with 0.2 M sodium chloride. Elution with higher sodium chloride concentrations did not result in further GST fractions being eluted. The GST pool was buffer exchanged on a G-75 gel filtration column. Again, only one peak of GST activity was observed and the GST pool was further purified using a GSH-Agarose affinity column (Sigma-Aldrich Company Ltd). After washing the column, bound protein was eluted with 10 M GSH as a single peak of GST activity. No further GST activity could be removed from this column by increasing the GSH concentration. SDS-PAGE analysis of the post-affinity column GST pool revealed one, 27.5 kDa, polypeptide. This compares well with molecular masses quoted for other plant GSTs and also with a value for a polypeptide detected in herbicide-susceptible black-grass extracts using antisera raised to wheat GSTs.[7] Purification of GSTs from the resistant biotype Peldon, which shows approximately double the GST activity of susceptible biotypes, resulted in a similar elution pattern for each column. However, SDS-PAGE analysis of the final post-affinity GST pool revealed two polypeptides (27.5 kDa and 30 kDa). The smaller polypeptide has a molecular mass similar to that of the susceptible biotype's GST. The same amount of starting material was used in both extracts and the extract from Peldon resulted in a higher yield of this polypeptide. The larger polypeptide has a similar molecular mass to a polypeptide identified in Peldon extracts using anti-wheat GST antibodies.[7] Although no GST activity can currently be attributed to the larger polypeptide, we speculate that this is a GST subunit that does not occur in susceptible biotypes. The presence of this, along with the increased amount of the smaller polypeptide, may be responsible for the increased GST activities observed in the Peldon biotype.

The effect of both atrazine and CTU on GST activity against CDNB was assessed using crude black-grass protein extracts. GST assays were carried out as previously described[8] using CDNB and GSH concentrations of 1 mM. The results of this study are shown in Table 1.

Herbicides, dissolved in acetone, were added to a final concentration of 1 mM in the assay mixture and control samples contained similar volumes of acetone. Both herbicides were demonstrated to reduce GST activity after pre-incubation with the enzyme extract for 30 min. CTU also reduced GST activity in the absence of pre-incubation, whereas atrazine had no effect. Although these results do not prove that GSTs from black-grass can utilise atrazine or CTU as substrates, the observed reduction in activities demonstrates that both herbicides can affect the enzymic catalysis of CDNB-GSH conjugation. This reduction of GST activity may involve competition at the active site of the enzyme. Atrazine has previously been reported to be a substrate for GSTs from the weed *Abutilon theophrasti* (L) Medic[2] and GSTs have been implicated in both crop

Table 1. Effects of herbicides on in-vitro GST activities in crude extracts of Herbiseed (susceptible) and Peldon (resistant) black-grass biotypes

Biotype	*Activity with no pre-incubation*[a]			*Activity with pre-incubation for 30 min*[a]		
	Control	*Atrazine*	*Chlorotoluron*	*Control*	*Atrazine*	*Chlorotoluron*
Herbiseed	26.1(±2.1)	20.9(±0.3)	14.6(±2.5)	27.6(±2.4)	2.7(±1.5)	13.5(±1.0)
Peldon	76.0(±1.0)	75.4(±3.2)	68.7(±0.6)	64.1(±0.7)	38.3(±0.6)	47.5(±0.9)

[a] Activity expressed as nmol l-chloro-2,4-dinitrobenzene (CDNB) min^{-1} mg^{-1} total protein.

tolerance[9] and herbicide resistance[8] to a variety of other herbicides.

In conclusion, purification of a GST from susceptible black-grass revealed one polypeptide, whilst purification from a resistant black-grass biotype, which had approximately double the GST activity of susceptible biotypes, resulted in two polypeptides. The additional polypeptide had a slightly higher molecular mass and, although GST activity cannot be attributed to this polypeptide, it was eluted from a GSH affinity column with a peak of GST activity. A study of crude enzyme extracts of black-grass revealed that both atrazine and CTU can affect the enzymic conjugation of GSH with the artificial GST substrate CDNB. These observations will allow further study of herbicides' interaction with GST activity, and the role of GSTs in herbicide resistance.

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REFERENCES

1 Marrs KA, The functions and regulation of glutathione *S*-transferases in plants. *Annu Rev Plant Physiol Plant Mol Biol* **47**:127–158 (1996).

2 Anderson MP and Gronwald JW, Atrazine resistance in a velvetleaf (*Abutilon theophrasti*) biotype due to enhanced glutathione *S*-transferase activity. *Plant Physiol* **96**:104–109 (1991).

3 Moss SR and Cussans GW, Variability in the susceptibility of *Alopecurus myosuroides* (black-grass) to chlorotoluron and isoproturon. *Asps Appl Biol* **9**, *The Biology and Control of Weeds in Cereals*. pp 91–98 (1985)

4 Moss SR and Cussans GW, The development of herbicide-resistance populations of *Alopecurus myosuroides* (black-grass), in: *Herbicide Resistance in Weeds and Crops*. ed by Caseley JC, Cussans GW and Atkin RK, Butterworth-Heinemann pp 45–55 (1991)

5 Sharples CR, Hull MR and Cobb AH, The effect of herbicide safeners on chlorotoluron susceptible and resistant black-grass (*Alopecurus myosuroides*). *Proc Brighton Crop. Prot Conf – Weeds*. pp 359–360 (1995).

6 Reade JPH and Cobb AH, Purification and characterisation of a GST from the weed black-grass (*Alopecurus myosuroides* Huds.). *Pestic Sci* (submitted).

7 Cummins I, Moss S, Cole DJ and Edwards R, Glutathione transferases in herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). *Pestic Sci* **51**:244–250 (1997).

8 Sharples CR, Hull MR and Cobb AH, The effects of herbicide safeners on glutathione *S*-transferase activity and photosynthesis in black-grass (*Alopecurus myosuroides* Huds) susceptible and resistant to chlorotoluron. *Pestic Sci* (submitted).

9 Dixon D, Cole DJ and Edwards R, Characterisation of multiple glutathione transferases containing the GST I subunit with activities towards herbicide substrates in maize (*Zea mays*). *Pestic Sci* **50**:72–82 (1997).

Synthesis and insecticidal activity of CGA 293′343 – a novel broad-spectrum insecticide

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Abstract: CGA 293′343 is a novel broad-spectrum insecticide currently under world-wide development by Novartis Crop Protection. CGA 293′343 belongs to a new class of highly active compounds – the neonicotinoids – and provides excellent control of a wide variety of commercially important pests. It possesses contact, stomach and systemic activity. The long-lasting residual effect is a special benefit of this compound. In general, CGA 293′343 shows biological activity in the laboratory equal to or better than the neonicotinoids so far introduced to the market. Synthetic aspects, structure–activity relationships and the biological profile are discussed.

Keywords: CGA 293′343; thiamethoxam; neonicotinoid insecticide; synthesis; [1,3,5]oxadiazinane derivatives; insecticidal activity; structure–activity relationships

1 INTRODUCTION

Neonicotinoids[1] are a novel and distinct class of insecticides. They combine selective activity against insects with a favourable safety profile, and possess contact, stomach and systemic activity, making these compounds appropriate for foliar, granular and seed treatment application. Neonicotinoids act at the nicotinic acetylcholine receptor.[2] This mode of action has so far not been broadly used for insecticides, and consequently neonicotinoids are important for controlling insects resistant to other commonly used insecticides such as organophosphates, carbamates, and pyrethroids. Imidacloprid (**1**; Fig 1)[3] was the first neonicotinoid and was introduced to the market by Bayer in 1991. As second and third neonicotinoids of the subclass chloronicotinyl compounds, nitenpyram **2**[4] from Takeda and acetamiprid **3**[5] from Nippon Soda were brought to the market in 1995 and 1996, respectively.

The extremely high activity and the unique properties of these compounds encouraged us to initiate a synthetic research project in this area. Our first attempts resulted in the synthesis of acyclic nitroenamine, cyanoamidine and nitroamidine derivatives.[6–8] Then we developed a molecular-modelling-based approach for the design of novel structural types of neonicotinoids.[9] However, the real breakthrough was achieved in 1991 with the discovery of nitroimino-[1,3,5]oxadiazinane derivatives.[10] After optimisation of this chemistry, CGA 293′343 (Fig 1) was identified as the best compound and subsequently selected for development.

CGA 293′343 (ISO draft common name: thiamethoxam) has exceptional insecticidal activity

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